(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 June 2002 (13.06.2002)

PCT

(10) International Publication Number
WO 02/46451 A2

(51) International Patent Classification[7]: C12Q 1/00

(21) International Application Number: PCT/US01/50890

(22) International Filing Date: 25 October 2001 (25.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/697,225 26 October 2000 (26.10.2000) US

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/46451 A2

(54) Title: METHODSFOR THE IDENTIFICATION OF INHIBITORS OF CYSTEINE SYHTHASE EXPRESSION OR ACTIVITY IN PLANTS

(57) Abstract: The present inventors have discovered that cysteine synthase is essential for plant growth. Specially, the inhibition of cysteine synthase gene expression in plant seedlings results in severe developmental abnormalities. Thus, cysteine synthase can be used as a target for the identification of herbicides. Accordingly, the present invention provides methods for the identification of compounds that inhibit cysteine synthase expression or activity, comprising: contacting a compound with a cysteine synthase and detecting the presence and/or absence of binding between said compound and said a cysteine synthase, or detecting a decrease in cysteine synthase expression or activity. The methods of the invention are useful for the identification of herbicides.

METHODS FOR THE IDENTIFICATION OF INHIBITORS OF CYSTEINE SYNTHASE EXPRESSION OR ACTIVITY IN PLANTS

FIELD OF THE INVENTION

The invention relates generally to plant molecular biology. In particular, the invention relates to methods for the identification of herbicides.

BACKGROUND OF THE INVENTION

Cysteine synthase (EC 4.2.99.8) is an enzyme involved in the final step of cysteine biosynthesis and catalyzes the formation of the amino acid L-cysteine from O-acetyl-L-serine (OAS) and inorganic sulfide. Cysteine synthase genes have been characterized from a number of plants, and in most cases consist of a small multigene family. At least ten different cysteine synthase genes have been identified in *Arabidopsis thaliana*. The *Arabidopsis* cysteine synthase gene, *AtcysC1*, encodes a mitochondrial protein having both cysteine synthase activity (EC 4.2.99.8) and β-cyanoalanine synthase activity (EC 4.4.1.9). Yamaguchi *et al.* (2000) *Plant Cell Physiol 41*:465-476.

To date, there has been no report or suggestion that cysteine synthase activity is essential for plant growth and development. Rather, there have been several reports that inhibition of cysteine synthase expression had no effect on plant growth. For example, constitutive antisense RNA expression of a spinach cytoplasmic cysteine synthase (CSase A) cDNA in tobacco plants results in decreased cysteine synthase activity, but did not damage transgenic plants. Saito (1994) *Plant Physiol 106*:887-

895. Furthermore, constitutive antisense RNA expression of a wheat cytoplasmic cysteine synthase (*cys1*) cDNA in tobacco plants had no effect on cysteine synthase activity or on the phenotype of the transgenic plant. Thus, the prior art has not suggested that cysteine synthase is essential for plant growth and development.

SUMMARY OF THE INVENTION

Surprisingly, the present inventors have discovered that antisense expression of cysteine synthase cDNA in *Arabidopsis* results in the production of white seeds that fail to germinate. Thus, the present inventors have discovered that cysteine synthase is essential for normal seed development and germination, and can be used as a target for the identification of herbicides. Accordingly, the present invention provides methods for the identification of compounds that inhibit cysteine synthase expression or activity, comprising: contacting a compound with a cysteine synthase and detecting the presence and/or absence of binding between said compound and said cysteine synthase, or detecting a decrease in cysteine synthase expression or activity. The methods of the invention are useful for the identification of herbicides.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "binding" refers to a noncovalent interaction that holds two molecules together. For example, two such molecules could be an enzyme and an inhibitor of that enzyme. Noncovalent interactions include hydrogen bonding, ionic interactions among charged groups, van der Waals interactions and hydrophobic interactions among nonpolar groups. One or more of these interactions can mediate the binding of two molecules to each other.

Cysteine is described as compound No. 2850 in The Merck Index, 12th Ed., Merck Research Laboratories, Whitehouse Station, NJ, 1996. The term cysteine is synonymous with L-cysteine, Cys, β-mercaptoalanine, (R)-2-amino-3-mercaptopropanoic acid, 2-amino-3-mercaptopropionic acid, α-amino-β-thiolpropionic acid, half-cysteine and thioserine.

As used herein, the term "cysteine synthase" (EC 4.2.99.8) is synonymous with "O-acetyl-L-serine (thiol) lyase", "O-acetylserine lyase", "O-acetyl-L-serine lyase", "O-acetyl-L-serine sulphydrylase" and "OAS sulfhydrylase". For the purposes of the invention, cysteine synthase refers to any enzyme that catalyzes the formation of amino acid L-cysteine and acetate from O-acetylserine and H_2S. Thus, the Arabidopsis enzyme referred to as referred to as cysteine synthase C (AtCS-C, Csase C) and encoded by *AtcysC1*, which has both beta-cyanoalanine synthase activity and cysteine synthase activity, is included within the meaning of "cysteine synthase".

The term "herbicide", as used herein, refers to a compound that may be used to kill or suppress the growth of at least one plant, plant cell, plant tissue or seed.

The term "inhibitor", as used herein, refers to a chemical substance that inactivates the enzymatic activity of cysteine synthase. The inhibitor may function by interacting directly with the enzyme, a cofactor of the enzyme, the substrate of the enzyme, or any combination thereof.

A polynucleotide may be "introduced" into a plant cell by any means, including transfection, transformation or transduction, electroporation, particle bombardment, agroinfection and the like. The introduced polynucleotide may be maintained in the cell stably if it is incorporated into a non-chromosomal autonomous replicon or integrated into the plant chromosome. Alternatively, the introduced polynucleotide may be present on an extra-chromosomal non-replicating vector and be transiently expressed or transiently active.

The "percent (%) sequence identity" between two polynucleotide or two polypeptide sequences is determined according to the either the BLAST program (Basic Local Alignment Search Tool; Altschul and Gish (1996) *Meth Enzymol 266*:460-480 and Altschul (1990) *J Mol Biol 215*:403-410) in the Wisconsin Genetics Software Package (Devereux *et al.* (1984) *Nucl Acid Res 12*:387), Genetics Computer Group (GCG), Madison, Wisconsin. (NCBI, Version 2.0.11, default settings) or using Smith Waterman Alignment (Smith and Waterman (1981) *Adv Appl Math 2*:482) as incorporated into GeneMatcher Plus™ (Paracel, Inc., http://www.paracel.com/html/genematcher.html; using the default settings and the version current at the time of filing). It is understood that for the purposes of

determining sequence identity when comparing a DNA sequence to an RNA sequence, a thymine nucleotide is equivalent to a uracil nucleotide.

"Plant" refers to whole plants, plant organs and tissues (e.g., stems, roots, ovules, stamens, leaves, embryos, meristematic regions, callus tissue, gametophytes, sporophytes, pollen, microspores and the like) seeds, plant cells and the progeny thereof.

By "polypeptide" is meant a chain of at least four amino acids joined by peptide bonds. The chain may be linear, branched, circular or combinations thereof. The polypeptides may contain amino acid analogs and other modifications, including, but not limited to glycosylated or phosphorylated residues.

The term "specific binding" refers to an interaction between cysteine synthase and a molecule or compound, wherein the interaction is dependent upon the primary amino acid sequence or the conformation of cysteine synthase.

As used herein, "sulfide" refers to inorganic sulfide or S^{2-}. The sulfide useful in the methods of the invention may be supplied by chemicals such as, but not limited to, hydrogen sulfide, sodium sulfide, potassium sulfide, rubidium sulfide, cesium sulfide, ammonium sulfide, beryllium sulfide, magnesium sulfide, calcium sulfide, strontium sulfide, barium sulfide and the like.

Embodiments of the Invention

The present inventors have discovered that inhibition of cysteine synthase gene expression strongly inhibits the development and germination of plant seeds. Thus, the inventors are the first to demonstrate that cysteine synthase is a target for herbicides.

Accordingly, the invention provides methods for identifying compounds that inhibit cysteine synthase gene expression or activity. Such methods include ligand binding assays, assays for enzyme activity and assays for cysteine synthase gene expression. Any compound that is a ligand for cysteine synthase, other than its substrates, O-acetyl-L-serine and sulfide, or cofactors may have herbicidal activity. For the purposes of the invention, "ligand" refers to a molecule that will bind to a site on a polypeptide. The compounds identified by the methods of the invention are useful for the modulation of plant growth and development.

Thus, in one embodiment, the invention provides a method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting a cysteine synthase with said compound; and

b) detecting the presence and/or absence of binding between said compound and said a cysteine synthase;

wherein binding indicates that said compound is a candidate for a herbicide.

By "cysteine synthase" is meant any enzyme that catalyzes the formation of cysteine from O-acetyl-serine and sulfide. The cysteine synthase may have the amino acid sequence of a naturally occuring cysteine synthase found in a plant, animal or microorganism, or may have an amino acid sequence derived from a naturally occuring sequence. Preferably the cysteine synthase is a plant cysteine synthase.

By "plant cysteine synthase" is meant an enzyme that can be found in at least one plant, and which catalyzes the formation of cysteine from O-acetyl-serine and sulfide. The cysteine synthase may be from any plant, including both monocots and dicots. In one embodiment, the cysteine synthase is an *Arabidopsis* cysteine synthase. *Arabidopsis* species include, but are not limited to, *Arabidopsis arenosa, Arabidopsis bursifolia, Arabidopsis cebennensis, Arabidopsis croatica, Arabidopsis griffithiana, Arabidopsis halleri, Arabidopsis himalaica, Arabidopsis korshinskyi, Arabidopsis lyrata, Arabidopsis neglecta, Arabidopsis pumila, Arabidopsis suecica, Arabidopsis thaliana* and *Arabidopsis wallichii*. Preferably, the *Arabidopsis* cysteine synthase is from *Arabidopsis thaliana*, more preferably from *Arabidopsis thaliana* strain Columbia.

The cDNAs and amino acid sequences for the *A. thaliana* cysteine synthases are reported at GenBank and Embase accession numbers: emb|X80376 (*Atoacly*; SEQ ID NO:1); emb|X84097 (*Atcys3A*; SEQ ID NO:2); emb|X81697 (*AtcysACS1*; SEQ ID NO:3); gb|AB024282 and emb|AJ010505 (*AtcysC1* or *oas5*; having both cysteine synthase and beta-cyanoalanine synthase activities; SEQ ID NOs: 4 and 5); gb|AB024284 (*AtcysD1* or *oas3*; SEQ ID NO:6); gb|X81973 (*mt ACS1*); gb|AB003041 (*cs26*); gb|AB02423 (*AtcysD2*); gb|X81698 (*cp ACS 1*); gb|AJ011044 (*oas6*); gb|AJ011976, gb|AJ011603, gb|AJ010505 (*oas5*), gb|BE039257 and gb|BE037810.

In addition, the sequences of cysteine synthase cDNAs and genes from a variety of other plants and organisms are publicly available. See, for example, gb|BE611445, gb|BE610004, gb|BE608380, gb|BE555595, gb|BE348173, gb|BE348127, gb|BE330806, gb|BE329795 and gb|BE020856 (*Glycine max* cysteine synthase cDNA); gb|AI563124 (watermelon cysteine synthase cDNA); gb|AF195239 (*Pyrus pyrifolia* cysteine synthase cDNA); gb|BE036966, gb|BE035521, gb|BE033421, gb|AI822724 and gb|AI822708 (*Mesembryanthemum crystallinum* cysteine synthase cDNA); gb|AI728743, gb|AI725847 and gb|AI725745 (*Gossypium hirsutum* cysteine synthase cDNA); gb|AW063019 (Sugar beet cysteine synthase cDNA); gb|AW164079 (*Lotus japonicus* cysteine synthase cDNA); gb|BE431668, gb|AW224722, gb|AW224770, gb|AW032102 and gb|AW031426 (*Lycopersicon esculentum* cysteine synthase); gb|D37963 and gb|D14722 (*Spinacia oleracea* cysteine synthase cDNA); gb|AI352787 (*Brassica napus* cysteine synthase cDNA); gb|AF044173 and gb|AF044172 (*Solanum tuberosum* cysteine synthase mRNA); gb|X85803 (*Zea mays* cysteine synthase cDNA); gb|AB040503 (*Allium tuberosum* cysteine synthase cDNA); gb|AF073695, gb|AF073696 and gb|AF073697 (*Oryza sativa* cysteine synthase *rcs1, rcs2* and *rcs3* cDNAs); gb|AW145239 (*Physcomitrella patens* cysteine synthase cDNA); gb|U19395 (*Emericella nidulans* cysteine synthase (*cysB*) gene); gb|Z95395 (*Streptococcus gordonii* cysteine synthase gene); gb|X59595 (*Salmonella typhimurium* cysteine synthase *cys M* gene); gb|AW871292 and AW871235 (*Meloidogyne incognita* cysteine synthase mRNA); gb|AB028631 (*Entamoeba dispar* cysteine synthase 1 mRNA); gb|AB028632 (*Entamoeba dispar* cysteine synthase 2 mRNA); gb|AB006900, gb|AB000266 and gb|AB000265 (*Entamoeba histolytica* cysteine synthase mRNA); gb|AE000329 (*Escherichia coli* cysteine synthase gene); gb|AF246333 (*Acidithiobacillus ferroxidans* cysteine synthase gene); gb|U93874 (*Bacillus subtilis* cysteine synthase); gb|AB028629 (*Clostridium perfringens* cysteine synthase); gb|AJ389044, gb|AJ389018 and gb|AJ388898 (*Medicago truncatula* cysteine synthase cDNA); gb|AF186381 (*Bacillus thermolevorans* cysteine synthase cDNA); and gb|AW720774 (*Chlamydomaonas reinhardtii* cysteine synthase cDNA). All of the above cysteine

synthase cDNA sequences may be used as probes to isolate cysteine synthase cDNAs or genes from additional organisms, and to synthesize cysteine synthase polypeptides.

In various embodiments, the cysteine synthase is from barnyard grass (*Echinochloa crus-galli*), crabgrass (*Digitaria sanguinalis*), green foxtail (*Setana viridis*), perennial ryegrass (*Lolium perenne*), hairy beggarticks (*Bidens pilosa*), nightshade (*Solanum nigrum*), smartweed (*Polygonum lapathifolium*), velvetleaf (*Abutilon theophrasti*), common lambsquarters (*Chenopodium album L.*), *Brachiara plantaginea, Cassia occidentalis, Ipomoea aristolochiaefolia, Ipomoea purpurea, Euphorbia heterophylla, Setaria spp, Amaranthus retroflexus, Sida spinosa, Xanthium strumarium* and the like.

Fragments of a plant cysteine synthase may be used in the methods of the invention. The fragments comprise at least 10 consecutive amino acids of a plant cysteine synthase. Preferably, the fragment comprises at least 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90 or at least 100 consecutive amino acids residues of a plant cysteine synthase. Most preferably, the fragment comprises at least 10 consecutive amino acid residues of an *Arabidopsis* cysteine synthase. Preferably, the fragment contains an amino acid sequence conserved among plant cysteine synthases. Such conserved fragments are identified in Rolland *et al.* (1993) *Biochem J 293*:829-833. Those skilled in the art could identify additional conserved fragments using sequence comparison software.

Polypeptides having at least 80% sequence identity with a plant cysteine synthase are also useful in the methods of the invention. Preferably, the sequence identity is at least 85%, more preferably the identity is at least 90%, most preferably the sequence identity is at least 95%.

In addition, it is preferred that the polypeptide has at least 50% of the activity of a plant cysteine synthase. More preferably, the polypeptide has at least 60%, at least 70%, at least 80% or at least 90% of the activity of a plant cysteine synthase. Most preferably, the polypeptide has at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the activity of the *A. thaliana* 'Columbia' cysteine synthase of SEQ ID NO:6.

Preferably, the activity of the polypeptide is compared to the activity of the *Arabidopsis* cysteine synthase polypeptide. For the purposes of the invention, cysteine synthase activity refers to the ability to convert O-acetyl-L-serine and sulfide

to L-cysteine and acetate. Methods for measuring cysteine synthase activity are known in the art and are described herein.

Thus, in another embodiment, the invention provides a method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting said compound with at least one polypeptide selected from the group consisting of: a plant cysteine synthase, a polypeptide comprising at least ten consecutive amino acids of a plant cysteine synthase, a polypeptide having at least 85% sequence identity with a plant cysteine synthase, and a polypeptide having at least 80% sequence identity with a plant cysteine synthase and at least 50% of the activity thereof; and

b) detecting the presence and/or absence of binding between said compound and said polypeptide;

wherein binding indicates that said compound is a candidate for a herbicide.

Any technique for detecting the binding of a ligand to its target may be used in the methods of the invention. Preferably, the ligand and target are combined in a buffer. Polypeptides and proteins that can reduce non-specific binding, such as BSA or protein extracts from cells that do not produce the target, may be included in binding assay.

Many methods for detecting the binding of a ligand to its target are known in the art, and include, but are not limited to the detection of an immobilized ligand-target complex or the detection of a change in the properties of a target when it is bound to a ligand. For example, in one embodiment, an array of immobilized candidate ligands is provided. The immobilized ligands are contacted with a cysteine synthase protein or a fragment or variant thereof, the unbound protein is removed and the bound cysteine synthase is detected. In a preferred embodiment, bound cysteine synthase is detected using a labeled binding partner, such as a labeled antibody. In a variation of this assay, cysteine synthase is labeled prior to contacting the immobilized candidate ligands. Preferred labels include fluorescent or radioactive moieties. Preferred detection methods include fluorescence correlation spectroscopy (FCS) and FCS-related confocal nanofluorimetric methods. See http://www.evotec.de/technology.

Once a compound is identified as a candidate for a herbicide, it can be tested for the ability to inhibit cysteine synthase enzyme activity. The compounds can be tested using either *in vitro* or cell based enzyme assays. Alternatively, a compound can be tested by applying it directly to a plant or plant cell, or expressing it therein, and monitoring the plant or plant cell for changes or decreases in growth, development, viability or alterations in gene expression.

Thus, in one embodiment, the invention provides a method for determining whether a compound identified as a herbicide candidate by an above method has herbicidal activity, comprising: contacting a plant or plant cells with said herbicide candidate and detecting the presence or absence of a decrease in the growth or viability of said plant or plant cells.

By decrease in growth, is meant that the herbicide candidate causes at least a 20% decrease in the growth of the plant or plant cells, as compared to the growth of the plants or plant cells in the absence of the herbicide candidate. By a decrease in viability is meant that at least 20% of the plants cells, or portion of the plant contacted with the herbicide candidate are nonviable. Preferably, the growth or viability will be at decreased by at least 40%. More preferably, the growth or viability will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring plant growth and cell viability are known to those skilled in the art. It is possible that a candidate compound may have herbicidal activity only for certain plants or certain plant species.

The ability of a compound to inhibit cysteine synthase activity can be detected using *in vitro* enzymatic assays in which the disappearance of a substrate or the appearance of a product is directly or indirectly detected. Cysteine synthase converts O-acetyl-L-serine and sulfide to cysteine and acetate. Methods for detection of these substrates and products, such as spectrophotometry, mass spectroscopy, thin layer chromatography (TLC), reverse phase HPLC and sulfide ion selective electrode assays are known to those skilled in the art. For example, cysteine can be measured by complexing with red ninhydrin (Gaitonde (1967) *Biochem J 104*:627-633), derivitaization of the thiol group and identification by HPLC (Fahey *et al.* (1987) *Meth Enzymol 143*:85-96; Cooper *et al.* (1987) *Meth Enzymol 143*:141-143), determination of pyruvic acid after coupling with cysteine desulphydrase (Wedding (1987) *Meth Enzymol 143*:29-31) and by protein binding assays (Smith *et al.* (1987)

Meth Enzymol 143:144-148). In a preferred embodiment, cysteine synthase activity is measured using a ninhydrin assay in which cysteine concentration is measured by quantitating red ninhydrin complex at 550-560 nm. See, Schmidt (1990) Methods in Plant Biochemistry Vol. 3, Chapter 21, Dey *et al.*, Eds, Academic Press, London.

In the sulfide electrode assay, sulfide and calomel electrodes are attached to a signal buffer that serves as an impedance match. The sulfide concentration can be calculated form the potential measured by the electrode. See Hara *et al.* (1990) *Protein Expr Purif 1*:70-76.

Thus, the invention provides a method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting O-acetyl-L-serine and sulfide with cysteine synthase;

b) contacting O-acetyl-L-serine and sulfide with cysteine synthase and said compound; and

c) determining the concentration of cysteine, acetate, O-acetyl-L-serine and/or sulfide after the contacting of steps (a) and (b).

If a candidate compound inhibits cysteine synthase activity, a higher concentration of the substrates (O-acetyl-L-serine and sulfide) and a lower level of the products (cysteine and acetate) will be detected in the presence of the candidate compound (step b) than in the absence of the compound (step a).

Preferably the cysteine synthase is a plant cysteine synthase. Enzymatically active fragments of a plant cysteine synthase are also useful in the methods of the invention. For example, a polypeptide comprising at least 100 consecutive amino acid residues of a plant cysteine synthase may be used in the methods of the invention. In addition, a polypeptide having at least 80%, 85%, 90%, 95%, 98% or at least 99% sequence identity with a plant cysteine synthase may be used in the methods of the invention. Also, polypeptides having at least 80% sequence identity with at least 15 consecutive amino acid residues of a plant cysteine synthase are also useful in the methods of the invention. Preferably, the polypeptide has at least 80% sequence identity with a plant cysteine synthase and at least 50%, 75%, 90% or at least 95% of the activity thereof.

Thus, the invention provides a method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting O-acetyl-L-serine and sulfide with a polypeptide selected from the group consisting of: a polypeptide having at least 85% sequence identity with a plant cysteine synthase, a polypeptide having at least 80% sequence identity with a plant cysteine synthase and at least 50% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of a plant cysteine synthase;

b) contacting O-acetyl-L-serine and sulfide with said polypeptide and said compound; and

c) determining the concentration of cysteine, acetate, O-acetyl-L-serine and/or sulfide after the contacting of steps (a) and (b).

Again, if a candidate compound inhibits cysteine synthase activity, a higher concentration of the substrate (O-acetyl-L-serine and sulfide) and a lower level of the product (cysteine and acetate) will be detected in the presence of the candidate compound (step b) than in the absence of the compound (step a).

For the *in vitro* enzymatic assays, cysteine synthase protein and derivatives thereof may be purified from a plant or may be recombinantly produced in and purified from a plant, bacteria, or eukaryotic cell culture. Preferably these proteins are produced using a baculovirus or *E. coli* expression system. See, for example, Saito *et al.* (1992) *Proc Natl Acad Sci 89*:8078-8082. Methods for the purification of cysteine synthase from potato tubers, bell pepper, spinach and *Salmonella typhimurium* are described in Ishizawa *et al.* (1991) *Plant and Cell Physiol 41*:200-208; Romer *et al.* (1992) *J Biol Chem 267*:17966-17970; Yamaguchi *et al.* (1998) *Bioscience, Biotechnology and Biochemistry* 62:501-507; and Hara *et al.* (1990) *Protein Expr Purif 1*:70-76, respectively. Other methods for the purification of cysteine synthase proteins and polypeptides are known to those skilled in the art.

As an alternative to *in vitro* assays, the invention also provides plant and plant cell based assays. In one embodiment, the invention provides a method for identifying a compound as a candidate for a herbicide, comprising:

a) measuring the expression of cysteine synthase in a plant or plant cell in the absence of said compound;

b) contacting a plant or plant cell with said compound and measuring the expression of cysteine synthase in said plant or plant cell;

c) comparing the expression of cysteine synthase in steps (a) and (b).

A reduction in cysteine synthase expression indicates that the compound is a herbicide candidate. In one embodiment, the plant or plant cell is an *Arabidopsis thaliana* plant or plant cell.

Expression of cysteine synthase can be measured by detecting cysteine synthase primary transcript or mRNA, cysteine synthase polypeptide or cysteine synthase enzymatic activity. Methods for detecting the expression of RNA and proteins are known to those skilled in the art. See, for example, *Current Protocols in Molecular Biology* Ausubel *et al.*, eds., Greene Publishing and Wiley-Interscience, New York, 1995. The method of detection is not critical to the invention. Methods for detecting cysteine synthase RNA include, but are not limited to amplification assays such as quantitative PCR, and/or hybridization assays such as Northern analysis, dot blots, slot blots, in-situ hybridization, transcriptional fusions using a cysteine synthase promoter fused to a reporter gene, bDNA assays and microarray assays.

Methods for detecting protein expression include, but are not limited to, immunodetection methods such as Western blots, His Tag and ELISA assays, polyacrylamide gel electrophoresis, mass spectroscopy and enzymatic assays. Also, any reporter gene system may be used to detect cysteine synthase protein expression. For detection using gene reporter systems, a polynucleotide encoding a reporter protein is fused in frame with cysteine synthase, so as to produce a chimeric polypeptide. Methods for using reporter systems are known to those skilled in the art. Examples of reporter genes include, but are not limited to, chloramphenicol acetyltransferase (Gorman *et al.* (1982) *Mol Cell Biol 2*:1104; Prost *et al.* (1986) Gene 45:107-111), β-galactosidase (Nolan *et al.* (1988) *Proc Natl Acad Sci USA 85*:2603-2607), alkaline phosphatase (Berger *et al.* (1988) *Gene 66*:10), luciferase (De Wet *et al.* (1987) *Mol Cell Biol 7*:725-737), β-glucuronidase (GUS), fluorescent proteins, chromogenic proteins and the like. Methods for detecting cysteine synthase activity are described above.

Chemicals, compounds or compositions identified by the above methods as modulators of cysteine synthase expression or activity can then be used to control plant growth. For example, compounds that inhibit plant growth can be applied to a plant or expressed in a plant, in order to prevent plant growth. Thus, the invention provides a method for inhibiting plant growth, comprising contacting a plant with a compound identified by the methods of the invention as having herbicidal activity.

Herbicides and herbicide candidates identified by the methods of the invention can be used to control the growth of undesired plants, including both monocots and dicots. Examples of undesired plants include, but are not limited to barnyard grass (*Echinochloa crus-galli*), crabgrass (*Digitaria sanguinalis*), green foxtail (*Setana viridis*), perennial ryegrass (*Lolium perenne*), hairy beggarticks (*Bidens pilosa*), nightshade (*Solanum nigrum*), smartweed (*Polygonum lapathifolium*), velvetleaf (*Abutilon theophrasti*), common lambsquarters (*Chenopodium album L.*), *Brachiara plantaginea, Cassia occidentalis, Ipomoea aristolochiaefolia, Ipomoea purpurea, Euphorbia heterophylla, Setaria spp, Amaranthus retroflexus, Sida spinosa, Xanthium strumarium* and the like.

EXPERIMENTAL

Plant Growth Conditions

Unless, otherwise indicated, all plants were grown Scotts Metro-Mix™ soil (the Scotts Company) or a similar soil mixture in an environmental growth room at 22°C, 65% humidity, 65% humidity and a light intensity of ~100 μ-E m^{-2} s^{-1} supplied over 16 hour day period.

Seed Sterilization

All seeds were surface sterilized before sowing onto phytagel plates using the following protocol.

1. Place approximately 20-30 seeds into a labeled 1.5 ml conical screw cap tube. Perform all remaining steps in a sterile hood using sterile technique.

2. Fill each tube with 1ml 70% ethanol and place on rotisserie for 5 minutes.

3. Carefully remove ethanol from each tube using a sterile plastic dropper; avoid removing any seeds.

4. Fill each tube with 1ml of 30% Clorox and 0.5% SDS solution and place on rotisserie for 10 minutes.

5. Carefully remove bleach/SDS solution.

6. Fill each tube with 1ml sterile dI H_2O; seeds should be stirred up by pipetting of water into tube. Carefully remove water. Repeat 3 to 5 times to ensure removal of Clorox/SDS solution.

7. Fill each tube with enough sterile dI H_2O for seed plating (~200-400 μl). Cap tube until ready to begin seed plating.

Plate Growth Assays

Surface sterilized seeds were sown onto plate containing 40 ml half strength sterile MS medium (no sucrose) and 1% Phytagel using the following protocol:

1. Using pipette man and 200 μl tip, carefully fill tip with seeds and 0.1% agarose solution. Place 10 seeds across the top of the plate, about ¼ in down from the top edge of the plate.

2. Place plate lid ¾ of the way over the plate and allow to dry for 30 minutes or until agarose solution is dry. It is important to allow agarose solution to dry completely before sealing up plates in order to prevent contamination.

3. Using sterile micropore tape, seal the edge of the plate where the top and bottom meet.

4. Place plates stored in a vertical rack in the dark at 4°C for three days.

5. Three days after sowing, the plates transferred into a growth chamber with a day and night temperature of 22 and 20 °C, respectively, 65% humidity and a light intensity of ~100 μ-E m^{-2} s^{-1} supplied over 16 hour day period.

6. Beginning on day 3, daily measurements are carried out to track the seedlings development until day 14. Seedlings are harvested on day 14 (or when root length reaches 6 cm) for root and rosette analysis.

Example 1

Construction of a Transgenic Plant expressing the Driver

The "Driver" is an artificial transcription factor comprising a chimera of the DNA-binding domain of the yeast GAL4 protein (amino acid residues 147) fused to two tandem activation domains of herpes simplex virus protein VP16 (amino acid residues 413-490). Schwechheimer *et al.* (1998) *Plant Mol Biol 36*:195-204. This chimeric driver is a transcriptional activator specific for promoters having GAL4 binding sites. Expression of the driver is controlled by two tandem copies of the constitutive CaMV 35S promoter.

The driver expression cassette was introduced into *Arabidopsis thaliana* by agroinfection. Transgenic plants that stably expressed the driver transcription factor were obtained.

Example 2

Construction of Cysteine Synthase Antisense Expression Cassettes in a Binary Vector

Portions of two *Arabidopsis thaliana* cysteine synthase cDNAs were ligated into the PacI /AscI sites of the *E.coli/Agrobacterium* binary vector PGT3.2 in the antisense orientation. This placed transcription of the cysteine synthase antisense RNAs under the control of an artificial promoter that is active only in the presence of the driver transcription factor described above. The artificial promoter contains four contiguous binding sites for the GAL4 transcriptional activator upstream of a minimal promoter comprising a TATA box.

The ligated DNA was transformed into *E.coli*. Kanamycin resistant clones were selected and purified. DNA was isolated from each clone and characterized by PCR and sequence analysis. pPG237 expresses an *A. thaliana* cysteine synthase antisense RNA that is complementary to 761/772 nucleotides of the *AtcysC1* mRNA (nucleotides 1348 to 578 of gb|AB024282) and to 760/772 of the Arabidopsis thaliana cysteine synthase reported as emb|AJ010505. pPG244 expresses an *A. thaliana* cysteine synthase antisense RNA that is complementary to 554/556 nucleotides of the *Atoacly* mRNA (nucleotides 1226 to 671 of emb|X80376), 552/556 nucleotides of the *Atcys3A* mRNA (nucleotides 1263 to 708 of

emb | X84097) and 532/562 nucleotides of the *AtcytACS1* mRNA (nucleotides 1259 to 708 of emb | X81697).

The antisense expression cassette and a constitutive barnase expression cassette are located between right and left T-DNA borders. Thus, the antisense expression cassettes can be transferred into a recipient plant cell by agroinfection.

Example 3

Transformation of *Agrobacterium* with the Target Expression Cassette

pPG237 and pPG244 were transformed into *Agrobacterium tumefaciens* by electroporation. Transformed *Agrobacterium* colonies were isolated using Basta selection. DNA was prepared from purified Basta resistant colonies and the inserts were amplified by PCR and sequenced to confirm sequence and orientation. The clones were stored as a frozen glycerol stock.

Example 4

Construction of an *Arabidopsis* cysteine synthase Antisense Target Plants

The cysteine synthase target expression cassettes were introduced into *Arabidopsis thaliana* wild-type plants by the following method. Five days prior to agroinfection, the primary inflorescence of *Arabidopsis* thaliana plants grown in 2.5 inch pots were clipped in order enhance the emergence of secondary bolts.

At two days prior to agroinfection, 5 ml LB broth (10 g/L Peptone, 5 g/L Yeast extract, 5 g/L NaCl, pH 7.0 plus 25 mg/L kanamycin added prior to use) was inoculated with a clonal glycerol stock of Agrobacterium carrying pPG237 or pPG244. The cultures were incubated overnight at 28°C at 250 rpm until the cells reached stationary phase. The following morning, 200 ml LB in a 500 ml flask was inoculated with 500 µl of the overnight culture and the cells were grown to stationary phase by overnight incubation at 28°C at 250 rpm. The cells were pelleted by centrifugation at 8000 rpm for 5 minutes. The supernatant was removed and excess media was removed by setting the centrifuge bottles upside down on a paper towel for several minutes. The cells were then resuspended in 500 ml infiltration medium (autoclaved 5% sucrose) and 250 µl/L Silwet L-77™ (84% polyalkyleneoxide

modified heptamethyltrisiloxane and 16% allyloxypolyethyleneglycol methyl ether), and transferred to a one liter beaker.

The previously clipped *Arabidopsis* plants were dipped into the Agrobacterium suspension so that all above ground parts were immersed and agitated gently for 10 seconds. The dipped plants were then cover with a tall clear plastic dome in order to maintain the humidity, and returned to the growth room. The following day, the dome was removed and the plants were grown under normal light conditions until mature seeds were produced. Mature seeds were collected and stored desiccated at 4 °C.

Transgenic *Arabidopsis* T1 seedlings were selected using glufosinate treatment. Approximately 70 mg seeds from an agrotransformed plant were mixed approximately 4:1 with sand and placed in a 2 ml screw cap cryo vial.

The surface of the seeds was sterilized using the chlorine gas method. Briefly, the open vials were placed in a vacuum desiccator in a safety hood. A glass beaker containing 200 ml 5.25% sodium hypochlorite solution was placed in the desiccator. Two ml concentrated HCl was added to the hypochlorite solution and the cover was placed on the desiccator. Vacuum was applied briefly to seal the dessicator, and the seeds were left in the desiccator overnight.

One vial of sterilized seeds was then sown in a cell of an 8 cell flat. The flat was covered with a dome, stored at 4°C for 3 days, and then transferred to a growth room. The domes were removed when the seedlings first emerged. After the emergence of the first primary leaves, the flat was sprayed uniformly with a 1:3000 dilution of LibertyTM (AgrEvo; 11.3% glufosinate) in water, 0.005% Silwet (50 µl/L) until the leaves were completely wetted. The spraying was repeated for the following two days.

Ten days after the first spraying resistant plants were transplanted to 2.5 inch round pots containing moistened sterile potting soil. The transplants were then sprayed with herbicide and returned to the growth room. These herbicide resistant plants represent stably transformed T1 plants. Mature T1 plants are then dried and harvested for T2 seeds.

Example 5
Effect of cysteine synthase Antisense Expression in *Arabidopsis* Seedlings

The cysteine synthase target plants from the transformed plant lines obtained in Example 4 were crossed with the *Arabidopsis* transgenic driver line described above. The resulting F1 seeds were then subjected to a PGI plate assay to observe seedling growth over a 2-week period. Seedlings were inspected daily for growth and development. During this period, approximately half of seeds derived from one of the pPG237 cysteine synthase antisense target lines developed abnormal cotyledons and showed reduced root growth. This pPG237 line also showed a 76% reduction in total leaf area and a 73% reduction in root length as compared to wild-type plants. One of the pPG244 cysteine synthase antisense target lines was white and failed to germinate, apparently representing an embryo lethal phenotype, while about half of the plants of a second line exhibited mild chlorosis and developmental abnormalities. The results are summarized in Table 1.

Table 1

Phenotypes of plants expressing cysteine synthase antisense RNA

Construct	No. Wild Type	No. Abnormal	χ^2 Value[a]	Probability[a]
pPG237	7	3	0.900	0.348
pPG244	4	6	0.400	0.527

[a]Chi-square and P values (0.05) were obtained to evaluate the hypothesis that chlorosis and wild-type phenotypes are segregating in a 1:1 ratio.

The clear 1:1 segregation ration observed demonstrates that the antisense expression of these genes encoding cysteine synthases results in the inhibition of germination and causes abnormal development. Thus, cysteine synthases are essential genes for normal plant growth and development. The fact that progeny derived from other lines selected from the pPG237 and pPG244 transformations did not exhibit any

abnormal phenotypes is not unexpected since it well known that antisense expression does not work equally well in all independently transformed lines containing the same construct.

Example 6

Assay for Inhibitors of Cysteine Synthase Activity

The enzymatic activity of the cysteine synthase of SEQ ID NO:6 is determined in the presence and absence of candidate inhibitors in a 0.5 ml reaction mixture containing 50 mM potassium phosphate, pH 8.0, 4 mM sodium sulfide, 12.5 mM O-acetyl-L-serine and 10 μg cysteine synthase. The reaction mixture is incubated at 30oC for thirty minutes and terminated by the addition of 0.1 ml 7.5% TCA. The amount of cysteine in the resulting mixture is determined spectrophotometrically at 560 nm by the acid-ninhydrin method of Gaitonde (1967) *Biochem J 104*:627-633.

While the foregoing describes certain embodiments of the invention, it will be understood by those skilled in the art that variations and modifications may be made and still fall within the scope of the invention.

CLAIMS

1. A method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting a cysteine synthase with said compound; and

b) detecting the presence and/or absence of binding between said compound and said a cysteine synthase;

wherein binding indicates that said compound is a candidate for a herbicide.

2. The method of claim 1, wherein said cysteine synthase is a plant cysteine synthase.

3. The method of claim 2, wherein said cysteine synthase is an *Arabidopsis* cysteine synthase.

4. The method of claim 3, wherein said *Arabidopsis* cysteine synthase is a protein selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6.

5. A method for determining whether a compound identified as a herbicide candidate by the method of claim 1 has herbicidal activity, comprising: contacting a plant or plant cells with said herbicide candidate and detecting the presence or absence of a decrease in growth or viability of said plant or plant cells.

6. A method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting said compound with at least one polypeptide selected from the group consisting of: an amino acid sequence comprising at least ten consecutive amino acids of a plant cysteine synthase, an amino acid sequence having at least 85% sequence identity with a plant cysteine synthase, and an amino acid sequence having at least 80% sequence identity with a plant cysteine synthase and at least 50% of the activity thereof; and

b) detecting the presence and/or absence of binding between said compound and said polypeptide;

wherein binding indicates that said compound is a candidate for a herbicide.

7. A method for determining whether a compound identified as a herbicide candidate by the method of claim 6 has herbicidal activity, comprising: contacting a plant or plant cells with said herbicide candidate and detecting the presence or absence of a decrease in growth or viability of said plant or plant cells.

8. A method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting O-acetyl-L-serine and sulfide with a cysteine synthase in the absence of said compound;

b) contacting O-acetyl-L-serine and sulfide with a cysteine synthase in the presence of said compound;

c) determining the concentration of O-acetyl-L-serine, sulfide, cysteine and/or acetate after the contacting of steps (a) and (b).

9. The method of claim 8, wherein said cysteine synthase is a plant cysteine synthase.

10. The method of claim 9, wherein said cysteine synthase is an *Arabidopsis* cysteine synthase.

11. The method of claim 10, wherein said *Arabidopsis* cysteine synthase is a protein selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6.

12. A method for determining whether a compound identified as a herbicide candidate by the method of claim 8 has herbicidal activity, comprising: contacting a plant or plant cells with said herbicide candidate and detecting the presence or absence of a decrease in growth or viability of said plant or plant cells.

13. A method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting O-acetyl-L-serine and sulfide with a polypeptide selected from the group consisting of: an amino acid sequence comprising at least 100 consecutive amino acids of a plant cysteine synthase, an amino acid sequence having at least 85% sequence identity with a plant cysteine synthase, and an amino acid sequence having at least 80% sequence identity with a plant cysteine synthase and at least 50% of the activity thereof, in the absence of said compound;

b) contacting O-acetyl-L-serine and sulfide with said polypeptide in the presence of said compound;

c) determining the concentration of O-acetyl-L-serine, sulfide, cysteine and/or acetate remaining after the contacting of steps (a) and (b).

14. A method for determining whether a compound identified as a herbicide candidate by the method of claim 13 has herbicidal activity, comprising: contacting a plant or plant cells with said herbicide candidate and detecting the presence or absence of a decrease in growth or viability of said plant or plant cells.

15. A method for identifying a compound as a candidate for a herbicide, comprising:

a) measuring the expression of cysteine synthase in a plant or plant cell in the absence of said compound;

b) contacting a plant or plant cell with said compound and measuring the expression of cysteine synthase in said plant or plant cell;

c) comparing the expression of cysteine synthase in steps (a) and (b).

16. The method of claim 15 wherein said plant or plant cell is an *Arabidopsis* plant or plant cell.

17. The method of claim 15, wherein the expression of cysteine synthase is measured by detecting cysteine synthase mRNA.

18. The method of claim 15, wherein the expression of cysteine synthase is measured by detecting cysteine synthase polypeptide.

19. A method for determining whether a compound identified as a herbicide candidate by the method of claim 15 has herbicidal activity, comprising: contacting a plant or plant cells with said herbicide candidate and detecting the presence or absence of a decrease in growth or viability of said plant or plant cells.

20. The method of claim 19 wherein said plant or plant cell is an *Arabidopsis* plant or plant cell.

SEQUENCE LISTING

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Zayed, Adel
Gorlach, Jorn
Boyes, Doug
Davis, Keith
Hamilton, Carol
Ascenzi, Robert
Hoffman, Neil

<120> METHODS FOR THE IDENTIFICATION OF INHIBITORS OF CYSTEINE SYNTHASE EXPRESSION OR ACTIVITY IN PLANTS

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